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The development of microfabricated arrays for DNA sequencing and analysis

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Microfabricated arrays of immobilized oligodeoxynucleotide probes are proving to be a powerful tool for rapidly generating sequence data via hybridization. These arrays are made either by immobilization of the probe post-synthetically, or by *in situ* synthesis of the probe. Hybridization of the target is easily achieved on the arrays, with analysis proceeding either by direct detection, or through enzyme-mediated detection; analysis of the hybridization pattern yields sequence information about the target. Such facile and rapid data acquisition will assist the challenging task of sequencing the human genome, and also will lead to a new generation of diagnostic assays.

To complete the sequencing of the entire human genome, and to take advantage of the information gained, it is desirable to greatly increase the rate of DNA-sequence acquisition. The process of diagnosing genetic diseases would also benefit from a convenient method of sequence analysis. One way to obtain sequence information relatively quickly involves array hybridization detection. In such a system, a sample of unknown target DNA is applied to an ordered array of immobilized oligodeoxynucleotide probes, and the hybridization pattern is analysed to

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produce many pieces of sequence information simultaneously (Fig. 1a; Refs 1–4). The analysis time of these multiplexed hybridization assays is significantly decreased compared to standard sequencing systems that rely on gel based assays.

DNA arrays are leading to a family of new analytical techniques. The first analytical method, called sequencing by hybridization (SBH), to arise from an array of immobilized probes is simple hybridization detection to determine the sequence of the hybridized strand[2,3]. SBH is limited by the fact that base-pair mismatches in the hybrid may not be discriminated very well, and modifications must be incorporated to alleviate this problem. A refinement of this procedure, called positional SBH (PSBH), uses duplex probes containing 3′ single-stranded overhangs to capture the target, followed by enzymatic ligation of the target to the duplex probe (Fig. 1b), which serves to minimize mismatches[5]. This ligation detection has been modified further to incorporate an extension of the immobilized probe by using DNA polymerase, which additionally minimizes mismatches in the capture (Fig. 1c; Ref. 6). Another modification of multiplex hybridizations involves the capture of target strands, extension of the probe with labeled dideoxynucleoside triphosphates (ddNTPs), and analysis to reveal the nature of the extended base (Fig. 1d; Ref. 7). This method, termed genetic-bit analysis, has been used to detect single-nucleotide polymorphisms. It seems obvious from all these derivatives of array hybridization assays that have already been developed, that it will be possible to use microfabricated arrays in diagnostic assays, as well as to produce large quantities of sequence data.

Implementation of multiplexed hybridization procedures necessitates the development of arrays for the attachment of DNA, and for subsequent analysis. For successful performance, the immobilized DNA must be stable and not desorb during hybridization, washing, or analysis. The density of the immobilized oligodeoxynucleotide must be sufficient for the ensuing analyses; however, there must be minimal non-specific binding of non-target DNA to the surface. The immobilization process should not interfere with the ability of immobilized probes to hybridize; therefore, it is often best for only one point, ideally a terminus, of the DNA to be immobilized. Microparticles often meet many of the criteria for the immobilization of DNA, but they are not be amenable to the array format. Strategies for immobilizing probes on beads can, however, serve as good models in the development of arrays. The process of manufacturing these arrays for basic research and for industrial use is another obstacle in the development of microfabricated arrays. Once the arrays have been made, it is necessary to determine whether a complementary sequence can hybridize to the probe. This process can be monitored by various means, including by enzyme-based detection, as well as by direct methods such as fluorescence, luminescence and radiolabeling. Once the array has been constructed, and hybridization has successfully been achieved, analysis of the hybridization pattern on the array can yield a plethora of sequence information about the target.

Array development

DNA attachment to surfaces

Simple hybridization assays for sequence analysis and diagnostics can be carried out on beads, microtiter plates, or membranes; however, to scale these procedures to the more convenient, densely packed array format, facile methods must be available for the manufacture of DNA chips. In the development of such chips, a number of approaches have been examined as model formats; these include the immobilization of DNA on beads, microtiter plates and membranes.

In considering the model formats and the eventual development of arrays, there are essentially two methods of immobilization: post-synthetic attachment and *in situ* synthesis. The former method involves standard automated synthesis of oligodeoxynucleotides, removal of the strands from the support and immobilization on the surface of a bead, or at specific sites on the surface of a membrane or chip, by non-covalent or covalent binding. In the latter method, the desired oligodeoxynucleotide is synthesized directly on the support that is to be used for subsequent analysis.

Immobilizations on beads

Although the immobilization of DNA probes on beads will probably not be used in multiplexed hybridization assays, because beads are often easier to manipulate than chips, the procedures can provide data on general immobilization techniques, and aid the development of DNA arrays. Table 1 documents the numerous techniques that have been investigated in attempts to conjugate DNA to beads. One such method uses the biotin–streptavidin binding system; this is a non-covalent interaction, but the interaction is relatively strong, and may be considered equal in strength to a covalent interaction. Streptavidin-coated magnetic beads (Dynal, Oslo, Norway) are used to capture terminally labeled biotinylated oligodeoxynucleotides. The streptavidin–biotin interaction is stable enough to withstand stringent washing conditions; hybridization on the beads can proceed with minimal non-specific interactions to the beads; and the biotin–streptavidin complex is sufficiently stable that it is not cleaved by the laser pulses used in matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry[8]. A similar biotin–streptavidin system has been used in a microtiter plate assay and in an array format.

There are many methods that can be used to covalently bind DNA to beads (Table 1). Three of these methods make use of carbodiimide-mediated coupling to produce a covalent bond between the DNA and the bead: in one case, hydrazide-coated beads are bound to a carboxy-modified oligodeoxynucleotide[9]; similarly, amino-modified oligonucleotides are bound to carboxyl groups on the surface of controlled-pore glass (CPG) beads[10] or magnetic polystyrene beads[11]. Carbodiimide-mediated coupling has subsequently

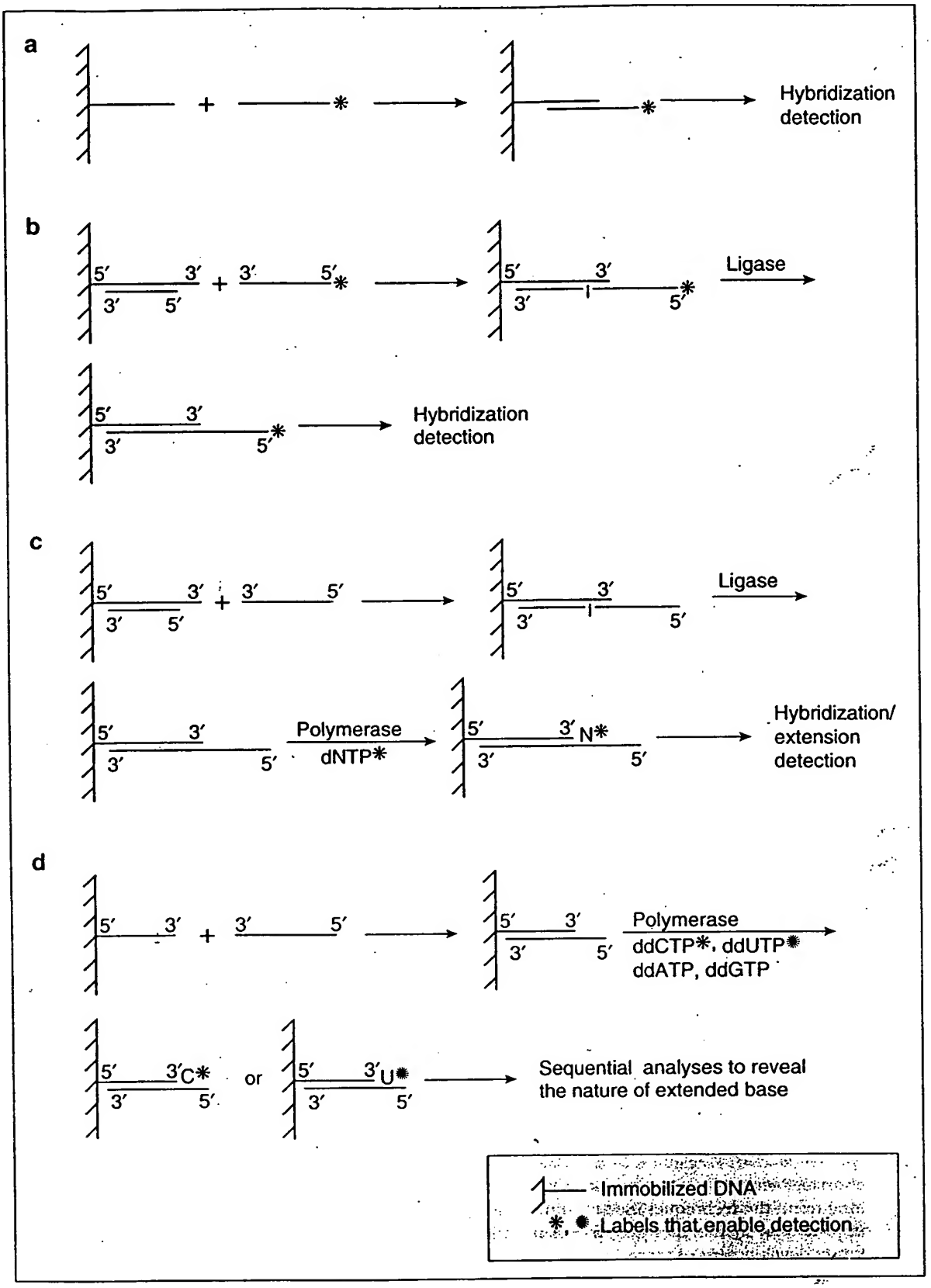

Figure 1

Immobilized DNA probes for (a) direct hybridization detection, (b) hybridization detection using ligation, (c) hybridization detection using ligation and extension, and (d) genetic-bit analysis. Abbreviations: ddATP, 2′,3′-dideoxyadenosine 5′-triphosphate; ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; ddUTP, 2′,3′-dideoxyuridine 5′-triphosphate; dNTP, deoxynucleoside triphosphate.

Table 1. Immobilization of DNA on beads that may serve as models for eventual array development

Bead	Oligodeoxynucleotide	Coupling agent	Amount of DNA immobilized (pmol mg^{-1} of bead)
Streptavidin-coated magnetic polystyrene bead	Biotinylated oligomer	–	200
Hydrazide-coated latex bead	COOH-oligomer	EDC	500
COOH-coated CPG bead	NH_2-oligomer	EDC	0.4
COOH-coated magnetic polystyrene bead	NH_2-oligomer	EDC	75
Epoxide-coated CPG bead	*In situ* synthesis	Standard 'PC'	46 000

Abbreviations: CPG, controlled-pore glass; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; 'PC', phosphoramidate chemistry.

been used in the development of arrays by attaching DNA to microtiter plates and membranes.

The last bead-immobilization technique shown in Table 1, which serves as a model for array development, makes use of *in situ* synthesis. Currently, there are two ways in which an oligodeoxynucleotide can be synthesized *in situ*, but only one of these methods has been carried out on beads. This method uses standard phosphoramidite chemistry; however, the linker that is used to attach the 3′ base to the support[12] is formed by first treating CPG beads with 3-glycidoxypropyltrimethoxysilane to place an epoxide functionality on the surface. The epoxide is then reacted with a diol, to produce an alkyl hydroxyl from which standard phosphoramidite chemistry can proceed to build the oligodeoxynucleotide. The linker is relatively stable to ammonia deprotection, ultimately resulting in an average of 46 nmol DNA mg^{-1} of bead.

Immobilizations on microtiter plates and membranes

Owing to the size of the wells and the relatively large volume of reagents needed for washing, commercially available microtiter plates will probably not become the surface of choice for array hybridizations. However, as wells define each element of the array, and the plates are commonly made of durable materials, multiplexed hybridization assays can be easily performed on microtiter plates. By contrast, hybridization assays on membranes can be carried out with smaller amounts of reagents because the procedures are not limited by the size of commercially available plates. Membranes can be difficult to use in array hybridizations because most are fragile and not as durable as 'solid' surfaces such as glass or silicon. Regardless of the plausibility of either surface being utilized in array hybridizations for the long term, the techniques used to immobilize DNA on plates and membranes can serve as models for the future development of arrays.

Using non-covalent interactions, DNA can be bound to a 96-well polystyrene microtiter plate in the presence of a relatively high concentration of salt (~500 mM NaCl; Ref. 13), or in the presence of a cationic detergent that possesses a hydrophobic 'tail' and a positively charged 'head'[7]. In both cases, the passive immobilization is sufficient for hybridization assays on microtiter plates. In another example of non-covalent immobilization, microtiter plates are coated with streptavidin, and these are subsequently used to capture biotinylated PCR products[14]. The PCR products bound to the plate can be analysed colorimetrically.

Some immobilization methods bind DNA to surfaces by simply making use of the reactivity of the nucleobases. In the first report of such binding, ultraviolet (UV) light was used to activate the thymine residues of the oligodeoxynucleotide, which were then covalently coupled to the primary amino groups of a nylon membrane[15]. It was believed, however, that this method of immobilization interfered with the oligonucleotide's ability to hybridize and discriminate single base-pair mismatches because many of the nucleobases are involved in the immobilization process. Therefore, the method was modified so that a long thymidine homopolymer tail [poly(dT)] was added to the oligodeoxynucleotides to be immobilized[16]. This poly(dT) tail is the preferential target for UV crosslinking to the nylon membrane, leaving the probe portion of the oligodeoxynucleotide free for hybridization. This method has proved to be relatively successful, and is still used for reverse dot-blots. A modification of UV crosslinking to membranes has been examined, in which microtiter plates are used for immobilization[17]. Colorimetric hybridization detection of PCR products in the wells of the plates enables the simultaneous handling of numerous samples.

There are various chemical methods for binding DNA to a plate or membrane at a specific site on the molecule. For example, an amino-modified oligonucleotide can be bound to the carboxyl groups on nylon Biodyne C membranes (Pall Biosupport, East Hills, NJ, USA) through carbodiimide-mediated coupling[18]. Microtiter plates have also been investigated for their ability to covalently bind DNA in this manner; the 5′ phosphate group of *Hinf* I-digested lambda DNA has been coupled to the amino groups of CovaLink NH (Nunc A/S, Roskilde, Denmark) plates[19].

Another example of the development of microtiter plate arrays is based on the reactivity of amino acids.

In one such example, an amino-modified oligodeoxynucleotide was treated with a homobifunctional crosslinking agent; the reactive oligodeoxynucleotide was immobilized onto a microtiter plate that had been coated with poly-(Lys + Phe) (Ref. 20). Similarly, a poly-(Lys + Phe)-coated microtiter plate was reacted with an oligodeoxynucleotide that had been functionalized to contain a *p*-nitrophenyl ester[13]. In a modification of the same approach, the amino groups of a poly-(Lys + Phe) microtiter plate were reacted with a heterobifunctional crosslinker to place a maleimido-functional group on the surface[20]. This functional group was then reacted with a thiol-containing oligodeoxynucleotide to covalently link the DNA to the plate.

Immobilizations for microfabricated arrays

Table 2 indicates the techniques that have been developed for attaching DNA to surfaces, for potential use as microfabricated arrays for hybridization assays. For example, the biotin–streptavidin binding system has been used: an amino-coated silicon wafer was reacted with the *N*-hydroxysuccinimido-ester of biotin, and this was complexed with streptavidin. Biotinylated oligonucleotides were bound to the surface at a concentration of ~20 fmol DNA mm^{-2} (M. J. O'Donnell-Maloney, H. K. Köster and C. R. Cantor, unpublished).

Three post-synthesis, covalent immobilization techniques are shown in Table 2. In the first case, an amino-group was attached to the surface of a glass slide using 3-aminopropyltrimethoxysilane; this amino group was then coupled to 1,4-phenylene diisothiocyanate to produce an amine-reactive surface. A 5′ amino-modified oligodeoxynucleotide was reacted with the slide, and bound at a concentration of ~130 fmol DNA mm^{-2} (Ref. 21). In the second case, a silicon wafer was functionalized with 3-aminopropyltriethoxysilane to place an amino-group on the surface; the amino group was reacted with a heterobifunctional crosslinking agent to incorporate an iodoacetamido-group on the surface; an oligodeoxynucleotide containing a 3′ thiol was coupled to the iodoacetamido-surface, resulting in a concentration of oligodeoxynucleotide of 250 fmol DNA mm^{-2} (Ref. 22). Approximately 50% of the immobilized oligodeoxynucleotide is available for hybridization, and analysis by MALDI mass spectrometry revealed that the covalent linkage is stable to laser desorption. The third method of covalent DNA-immobilization on a surface, which has recently been explored, involved reacting a silicon wafer with 3-glycidoxypropyltrimethoxysilane to place an epoxide monolayer on the surface[23,24]. A 3′-terminal amino-modified oligodeoxynucleotide was then coupled to the surface by secondary amine formation resulting in a concentration of ~16 fmol DNA mm^{-2}.

DNA has been synthesized *in situ* on glass slides, in a manner analogous to *in situ* synthesis on CPG beads, to produce 110 fmol DNA mm^{-2} of surface[12]. The original procedure reported that it was necessary to perform the *in situ* synthesis manually on the glass slide, rather than using automation; however, more recently, a method has been devised to automate this procedure[25].

An additional method of synthesizing oligodeoxynucleotides *in situ* combines solid-phase chemistry, photolabile protecting groups and photolithography, and results in a set of spatially distinct and highly diverse chemical products. This method was originally devised for peptides, but can also be used for the synthesis of oligodeoxynucleotides[26]. It was possible to assemble on one glass surface all 65 536 different octanucleotides (4^8) in only 32 chemical steps[27]. In this method, light is used to cleave photolabile protecting groups from the surface, and the entire surface is then exposed to a phosphoramidite; coupling only takes place where the protecting groups have been removed. Exposure to illumination determines which regions of the support are activated for chemical coupling, and this process is controlled using a series of 'masks'. The cycles continue until a diverse set of 65 536 spatially defined oligodeoxynucleotides has been synthesized.

Manufacturing considerations

Many factors must be considered when designing microfabricated arrays for research purposes or industrial use: the density and stability of the immobilized oligodeoxynucleotide are major concerns; the desired

Table 2. Immobilization of DNA on 'solid' surfaces

Solid surface	Oligodeoxynucleotide	Coupling agent	Amount of DNA immobilized (fmol mm^{-2} of surface)
Streptavidin-coated silicon wafer	Biotinylated oligomer	–	20
Isothiocyanate-coated glass	NH_2-oligomer	–	130
Iodoacetamide-coated silicon wafer	SH-oligomer	–	250
Epoxide-coated silicon wafer	NH_2-oligomer	–	16
Epoxide-coated glass	*In situ* synthesis	Standard 'PC'	110
Amine-coated glass	*In situ* synthesis	Photolithography	nr

Abbreviations: nr, not reported; 'PC', phosphoramidate chemistry.

spot size of each element and the spacing between the elements are variable; the same sample can be placed on all elements of the array, or each element can contain a different sample; and the method of sample delivery to the array must also be addressed. In terms of the density and stability of the bound oligonucleotide, the researcher often has to choose whether simplicity in synthesis is more desirable than producing a highly stable bond between the immobilized probe and the surface. If bond stability is not of great concern, then non-covalent immobilization can be quite simple to achieve and is cost-effective, because modified oligonucleotides are not necessary. However, if a stronger bond between the oligodeoxynucleotide and the surface is needed to withstand stringent washes, or to withstand harsh conditions in analysis, then covalent immobilizations should be employed. In these cases, there is an optimal density of conjugated oligodeoxynucleotide on the surface at which charge and steric effects are minimal, but at which there is sufficient hybrid annealed for the ensuing applications.

To create an array on a surface by post-synthesis manipulation of DNA, the spatial distribution at which elements are placed must be considered. If an array is to be used for basic research purposes and a limited number of elements will suffice, then the coupling can be accomplished manually. For example, manual deposition of oligodeoxynucleotides on an isothiocyanate-derivatized glass slide can be facilitated by applying the solution in a spot pattern following a paper template underneath the slide[21]. Quite often, however, the number of elements and spatial distribution prohibit manual deposition. In addition, when a large number of arrays are needed for industrial use, manual deposition is not feasible; in these cases, deposition can be carried out robotically. This approach has been used to create an array of 96 elements in a 3.5×5.5 mm area on a glass slide[28], but further research is required for the manufacture of a large number of arrays for industrial applications.

Many of these manufacturing issues can be easily addressed if *in situ* synthesis is the chosen method of immobilization. For example, a full set of complements to a known sequence has been synthesized *in situ* on a 50×220 mm glass slide using a teflon reaction cell in an automated DNA synthesizer[25]. Photolithography has been used to assemble all 65 536 different octanucleotides (4^8) in only 32 chemical steps[27]. Despite the difficulty in monitoring the quality of each immobilized oligodeoxynucleotide, the only currently available array of more than 50 immobilized probes is made by this technology.

Hybridization analysis on arrays

Various methods are currently available for the analysis of hybridization patterns on arrays of immobilized probes; some rely on the use of enzymes to enable detection, while others detect hybridization directly. Regardless of the method chosen, array hybridizations are capable of producing data for basic research, as well as for genetic analysis.

Enzyme-assisted detection

The precursor to the current interest in microfabricated DNA arrays is the reverse dot-blot[16]. In this hybridization detection system, an array of DNA probes with poly(dT) tails is immobilized on nylon membranes using UV irradiation; biotinylated PCR products are hybridized to the membrane; streptavidin–horseradish peroxidase (HRP) is complexed to the biotin on the membrane; and hybridization is detected by a colorimetric reaction involving the oxidation of a colorless chromogen by HRP, yielding a red color wherever hybridization has occurred. This technique has been applied to *HLA-DQA* genotyping and to the detection of β-thalassemia mutations. In a modification of the reverse dot-blot technique, Biodyne C membranes are used to immobilize the probes[4] and, following hybridization of biotinylated PCR products and streptavidin–HRP complexation, a chemiluminescent detection system is used. Reverse hybridization analysis has also been developed into a microtiter plate format; the wells of the plate contain immobilized probes to which biotinylated PCR products may hybridize, and this is followed by enzyme-assisted colorimetric detection[17]. Such a plate format is suited to handling a large number of samples.

Variations in the enzyme-based detection system have also been explored. For example, PCR can be performed using one biotinylated primer, and a modified triphosphate (digoxigenin-11-dUTP) is incorporated during the reaction[14]. The products are captured on a streptavidin-coated microtiter plate, and an anti-digoxigenin antibody conjugated to alkaline phosphatase is added. Detection is achieved using colorimetric, fluorescent, or chemiluminescent substrates.

Genetic-bit analysis (Fig. 1d), which makes use of an array of immobilized DNA probes and enzyme-assisted hybridization detection, can be used for detecting single-nucleotide polymorphisms[7]. In this technique, specific fragments of genomic DNA that contain polymorphic sites are amplified by PCR using an unmodified primer and a phosphorothioate-containing primer. The PCR product is digested by T7 gene 6 exonuclease, rendering it single-stranded; the strand is captured by hybridization to an immobilized probe, which is designed to hybridize to the target adjacent to the polymorphic site of interest, in the wells of a microtiter plate. The 3′ end of the immobilized probe is extended by one base using a polymerase in the presence of one biotin-labeled, one fluorescein-labeled and two unmodified ddNTPs. If the labeled and unlabeled ddNTPs are reversed in a parallel experiment, because of the different haptenated-ddNTPs incorporated, two enzyme-assisted colorimetric assays can then be performed sequentially to reveal the nature of the extended base.

Direct detection

The hybridization detection method that is used most frequently involves radioactivity. Radiolabeled DNA is hybridized to an immobilized array of probes, and the hybridization pattern may be detected

autoradiographically[2] or using storage phosphor screen technology (phosphorimager)[29]. More recently, fluorescent reporter groups have been used for direct hybridization detection: PCR is performed using a biotinylated primer and a fluorescently labeled primer; the product is denatured on streptavidin-coated magnetic beads; the fluorescently labeled strand is hybridized to an array of immobilized probes on a glass slide; and the hybridization pattern is detected by fluorescence scanning[21]. This method has been used for the identification of polymorphisms from exon 4 of the human tyrosine gene.

Other methods of direct hybridization detection are becoming available. For example, it is now possible to use MALDI-TOF mass spectrometry to rapidly analyze duplex DNA with the oligonucleotide probes immobilized on streptavidin beads, CPG beads[8], or silicon wafers[22]. Analysis reveals that strand separation caused by the laser results in direct desorption and detection of the target strand, while the immobilized strand remains intact and bound to the support. A second method of hybridization detection uses a charge coupled device (CCD) to obtain data rapidly[23,24]. The ^{32}P-radiolabeled target is hybridized to an immobilized probe on a silicon wafer; the wafer is then placed upon the CCD surface and a signal is generated. Using this technique, hybridization signals can be obtained ~10-fold faster than by using a gas-phase detector or a phosphorimager, and 100-fold faster than by using autoradiography. In addition, this CCD-based detector is more amenable to miniaturization than the macro-sized detectors mentioned previously. A third method of hybridization analysis involves the use of two-dimensional optical wave guides[30]. In this method, DNA is immobilized on a glass slide and biotinylated target molecules are hybridized. An anti-biotin–selenium conjugate is then added to the slide, and the slide is illuminated by the evanescent wave of the wave guide. Light is scattered from the selenium labels, and the hybridization pattern is detected visually or using a CCD camera. The signal intensity is sufficient to allow real-time analysis of hybridization, as well as to allow monitoring DNA denaturation curves.

Future prospects

Array hybridization is a potentially powerful tool for producing large quantities of DNA-sequence information, and the development of this approach is continuing at a rapid pace. The production of a variety of different kinds of DNA arrays is essential for the advancement of these hybridization assays. It is also imperative to increase our understanding of hybridization itself, so that it is possible to eliminate the effects of non-specific binding, secondary structure and cross hybridization. Additionally, detection systems are being upgraded to produce more sequence data at a faster rate. However, in order to increase the amount of information that is obtained, it is also necessary to increase the sensitivity of the system; this can be achieved by various routes. First, if the amount of immobilized probe on the array can be increased then the amount of hybridized product will in turn be increased, thus producing a better signal in the detection system. Also, if the sensitivity of the detection system can be improved, more data can be obtained from each experiment because a higher density of smaller sample spots can be used. Even without future improvements, microfabricated arrays are already powerful tools for hybridization detection, and for producing fast and reliable sequencing data.

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